factor of 100 in favor of the canonical substrate compared to the non-canonical substrate. Similarly, a value less than 1 means that the enzyme discriminates in favor of the non-canonical substrate over the canonical substrate.

4. Finally, one compares the numerical value calculated in step 3 above for the wild-type (w.t.) enzyme with the value calculated in step 3 for the mutant enzyme. If the value calculated for the mutant enzyme is less than the value calculated for the wild-type enzyme, then the mutant enzyme "has a reduced discrimination for the non-canonical substrate compared to the wild-type enzyme." For example, if the values calculated in step 3 are 100 for the wild-type enzyme and 10 for the mutant enzyme, then the discrimination of the mutant enzyme in favor of the canonical substrate (or against the non-canonical substrate) is reduced 10-fold compared to the discrimination of the wild-type enzyme.

We have found that for wild-type T7 RNAP, the average of the k_{cat}/K_m values for the four common rNTPs (ATP, CTP, GTP & UTP) is about 120-fold larger than the average k_{cat}/K_m values for the four common dNTPs (dATP, dCTP, dGTP and dTTP); i.e., the wild-type enzyme discriminates by a factor of 120 for rNTPs vs. dNTPs. For the Y639F mutant enzyme, the average of the k_{cat}/K_m values for the four common rNTPs is only about 6-fold larger than the average k_{cat}/K_m values for the four common dNTPs. Thus, using the average k_{cat}/K_m values for these substrates, the Y639F mutant T7 RNAP enzyme has about a 20-fold reduced discrimination between dNTPs and rNTPs. However, it is recognized that the difference in discrimination between wild-type and mutant enzymes will vary depending on the non-canonical substrates and the

mutant enzymes used. Therefore, for the purposes of this invention, we herein define "a (mutant) nucleic acid polymerase (enzyme) with reduced discrimination between canonical and non-canonical nucleoside triphosphate substrates" as "a polymerase which has at least a 10-fold reduced discrimination compared to the corresponding wild-type enzyme for non-canonical nucleotides compared to canonical nucleotides, wherein the respective values for discrimination between canonical and non-canonical substrates is calculated using the average of the k_{cat}/K_m values for all four rNTPs and all four dNTPs."

By "T7-type RNA polymerases" we mean T7, T3, ΦI, ΦIIH, W31, ghl, Y, A1122, SP6 and mitochondrial RNAPs.

In General

There are many reasons to synthesize nucleic acid molecules containing at least one non-canonical nucleotide. For example, incorporation of a non-canonical nucleotide may make the synthetic nucleic acid more resistant and therefore, more stable, to a nuclease, such as a ribonuclease. Also, one may wish to incorporate one or more non-canonical nucleotides which, for example, change the nuclease digestion pattern so that the product nucleic acid is easier to detect or characterize. For example, because RNase A cleaves RNA only after C or U, replacement of one or both of these rNMPs by a dNMP or other non-canonical nucleotide that is resistant to cleavage by RNase A would alter the digestion pattern of the nucleic acid.

There are many uses for nucleic acids which have one or more of these properties, such as nuclease resistance. For example, nucleic acids containing at least one non-canonical nucleotide may have advantages for use as ribozymes, or as

nucleic acid molecules used for gene therapy, in a vaccine, in an antiviral composition, in an antimicrobial composition, in an anti-sense composition for regulating gene expression, in a composition for hybridization to a complementary nucleic acid, including as a primer, or as a probe for detection of a complementary nucleic acid for a variety of purposes.

Some nucleic acid molecules, such as those of mixed dNMP/rNMP composition, are highly useful for certain applications, but are presently difficult or impossible to produce on a practical scale. Thus, improved methods for synthesizing such nucleic acid molecules *in vitro* would be highly desirable. For example, probes of mixed DNA-RNA-DNA composition for the Cycling Probe Assay (Duck, P.G., et al., 1990) are currently made using difficult chemical methods.

We describe herein previously-unknown properties of T7-type RNA polymerases having a non-wild-type amino acid at specific positions within the polypeptides. We found that altering the amino acid at these specific positions results in mutant polymerases having at least a 10-fold reduced discrimination between 2'-deoxyribonucleoside-5'-triphosphates (dNTPs) and ribonucleoside-5'-triphosphates (rNTPs) as substrates in *in vitro* nucleic acid synthesis reactions compared to the corresponding wild-type enzymes. We found that these mutant polymerases also have reduced discrimination for other non-canonical nucleoside triphosphate (NTP) substrates, including 2',3'-dideoxy-ribonucleoside-5'-triphosphates (ddNTPs) and 2'-fluoro-nucleoside-5'-triphosphates (2'-F-NTPs). Based on knowledge of these novel properties, we have disclosed methods for using these mutant polymerases for producing nucleic acid molecules containing at least one non-canonical nucleotide